Yin and Yang of mitochondrial ROS in *Drosophila*

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Abstract

In this study, we test the hypothesis that *Drosophila* larvae producing mildly elevated levels of endogenous mitochondrial reactive oxygen species (ROS) benefit in stressful environmental conditions due to the priming of antioxidant responses. Reactive oxygen species (ROS) are produced as a by-product of oxidative phosphorylation and may be elevated when mutations decrease the efficiency of ATP production. In moderation, ROS are necessary for cell signaling and organismal health, but in excess can damage DNA, proteins, and lipids. We utilize two *Drosophila melanogaster* strains (Dahomey and Alstonville) that share the same nuclear genetic background but differ in their mitochondrial DNA haplotypes. Previously, we reported that Dahomey larvae harboring the V161L ND4 mtDNA mutation have reduced proton pumping and higher levels of mitochondrial ROS than Alstonville larvae when they are fed a 1:2 protein: carbohydrate (P:C) diet. Here, we explore the potential for mitochondrial ROS to provide resistance to dietary stressors by feeding larvae 1:2 P:C food supplemented with ethanol or hydrogen peroxide (H_2O_2). When fed a diet supplemented with ethanol or H_2O_2, Dahomey develop more quickly than Alstonville into larger pupae, while Alstonville developed faster on the control. Dahomey larvae displayed higher antioxidant capacity than Alstonville on all diets, with mitochondrial H_2O_2 levels unchanged after the addition of stressors. Addition of stressors to the diet did not affect the mitochondrial functions of Dahomey larvae as measured by mitochondrial membrane potential, respiratory control ratio, or larval survival after bacterial challenge. In contrast, Alstonville larvae developed slower, had lower pupal weight, higher cytosolic H_2O_2, and had reduced mitochondrial functions. Further, Alstonville larvae fed the ethanol treated diet had lower survival after bacterial infection than those fed the control diet. Surprisingly, they had greater survival when fed diet with H_2O_2 indicating a mitotype by stressor interaction that influences the immune response. Overall, these data suggest that elevated mitochondrial ROS in

Dahomey can result in greater antioxidant capacity that prevents oxidative damage from exogenous stressors and may be a conserved response to high ethanol found in rotting fruit.

Keywords: *Drosophila*, mitochondrial DNA; Reactive oxygen species; Dietary stressors; Beneficial mutation

Abbreviations

GstE1, Glutathione S transferase E1; H_2O_2, hydrogen peroxide; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; P:C, Protein: Carbohydrate; RCR, Respiratory Control Ratio; ROS, Reactive Oxygen species; RIRR, ROS induced ROS release; *Sod2, Superoxide dismutase 2 (Mn).*

1. Introduction

Physiological stressors present strong selective pressures on insect populations, primarily through dysregulation of cell homeostasis. Determination of the physiological responses to chemical stressors is important, as exposure to these exogenous stressors is known to influence development time of insects (Bednarova et al., 2015) as the responses are energetically taxing (reviewed in Kodrik et al., 2015). The addition of industrial pollutants to diet has shown wide range of effects on insects including a reduction in pupal weight of *Panolis flammea* and *Bupalus piniarius* (Heliovaara et al., 1989), and increased larval mortality of *Lymantria monacha* (Mitterbock and Fuhrer, 1988). Interestingly, positive responses to environmental stressors have also been identified in insects. For example, *Acyrthosiphon pisum* displays faster growth and increased adult weight when exposed to low levels of sulfur dioxide (Warrington, 1987). Adult weight is positively correlated with larval weight, reproduction and longevity (Kingsolver and Huey, 2008) in a variety of insects including *Drosophila* (Yadav and Sharma, 2014), longicorn beetles (Wang et al., 2002), moths (Calvo and Molina, 2005), and leaf miners (Quiring and McNeil, 1984).

Specific genotypes of insects have been shown to differentially respond to environmental stressors (Clarke et al., 2009; Duan et al., 2001; Mpho et al., 2002; Polak et al., 2004; Ringwood et al., 2009), however response to exogenous stressors is not only influenced by the nuclear genotype. It has been demonstrated that mitochondrial DNA (mtDNA) haplotypes (mitotypes) respond differently to environmental stress including naphthalene, traffic pollution and pesticides (Chung et al., 2013; Colicino et al., 2014; Schizas et al., 2001; Wittkopp et al., 2013). While these studies have identified mitotype specific responses to environmental stressors, they have not identified a possible mechanism. Here we investigate the responses of the Dahomey and Alstonville *D. melanogaster* mitotypes to dietary stress. Dahomey harbors a mtDNA encoded non-synonymous V161L

ND4 mutation (Clancy, 2008) that is predicted to reduce proton pumping and has been shown to reduce ATP production and produce higher levels of reactive oxygen species (ROS) on a 1:2 protein: carbohydrate (P:C) diet (Aw et al., 2018).

Excess ethanol is a natural exogenous stressor (Chauhan and Chauhan, 2016; Logan-Garbisch et al., 2014; Service et al., 1985) and specific evolutionary response mechanisms may be conserved (Kong et al., 2010). Adult female *Drosophila* flies are known to follow ethanol plumes to locate ripe fruit suitable for oviposition (Hoffmann and Parsons, 1984). Larvae feed on rotting fruit that are in the process of fermenting and can face ethanol levels up to 7% (Gibson et al., 1981) before its eventual conversion to acetic acid. Exposure to ethanol has been shown to increase ROS levels in *D. melanogaster* (Niveditha et al., 2017), rats (Chen et al., 1997; Hamby-Mason et al., 1997) and yeast (Jing et al., 2018).

We predict that organisms will benefit from a flexible network of responses to reduce ROS levels below a detrimental level. Endogenous ROS is produced endogenously as a by-product of oxidative phosphorylation (OXPHOS) and may be elevated when mutations decrease the efficiency of ATP production (Aw et al., 2018; Vives-Bauza et al., 2006). Moderate levels of mitochondrial ROS are necessary for cell signaling and organismal health (Ristow and Schmeisser, 2014; Tal et al., 2009; Zarse et al., 2012). In excess, however, ROS can damage DNA (Biancini et al., 2015; Scott et al., 2014; Yan et al., 2014), proteins (Fedorova et al., 2014; Grimm et al., 2012), and lipids (Jaeschke and Ramachandran, 2018). High levels of mitochondrial ROS result in increased leak into the cytosol through the process termed “ROS induced ROS release” (RIRR) (Zorov et al., 2000; 2014). Such RIRR may maintain mildly elevated cytosolic ROS levels, prime the antioxidant response, and provide increased resistance to dietary stressors (Zarse et al., 2012). Differences in the response to elevated cytosolic ROS may occur between genotypes, generations, and sexes (Clark and Fucito, 1998; Hoffmann et al., 2001; Neckameyer and Nieto-Romero, 2015).

Antioxidants respond to increasing ROS levels. The antioxidant response is multifaceted, varying from detoxification of superoxide's, to repair of damaged tissue and lipids. SOD constitutes the first line of defense in the antioxidant enzyme network and is the primary scavenger of the ROS superoxide. The two main forms of SOD in eukaryotic organisms are manganese SOD, which is localized in the mitochondria, and copper – zinc SOD, which operates in the cytosol (Filograna et al., 2016; Phillips et al., 1989). Two antioxidant genes were assayed in this study, *superoxide dismutase 2* (*Mn*) (*Sod2*) and *glutathione S transferase E1* (*GstE1*). Sod2 acts in the mitochondria (Duttaroy et al., 2003), while GstE1 is localized to the cytosol and acts to detoxify lipids damaged by ROS (Sheehan et al., 2001).

We investigate mitochondrial membrane potential, respiratory control ratio (RCR) and response of two *Drosophila* mitotypes to bacterial challenge in response to exogenous stress. The mitochondrial membrane potential generated by proton pumps (Complexes I, III and IV) is an essential component in the process of energy storage during OXPHOS. Together with the proton gradient, membrane potential forms the transmembrane potential of hydrogen ions which is harnessed to produce ATP (Zorova et al., 2018). High membrane potential leads to high capacity for OXPHOS (Sakamuru et al., 2016). Low membrane potential may result from ROS damage produced as a by-product of decreasing OXPHOS efficiency (Suski et al., 2012). RCR measures the ability of mitochondria to return from maximal ATP generation to basal levels when coupled (Brand and Nicholls, 2011). High RCR indicates healthy OXPHOS capacity, while low RCR can indicate proton leak (Cheng et al., 2017). A bacterial challenge was undertaken as mitochondria are heavily involved in the innate immune response (West et al., 2015; West et al., 2011b). Innate immunity in *Drosophila* includes humoral and cellular factors. The humoral factors induce hemolymph coagulation, melanization and the synthesis of antimicrobial peptides (Cherry and Silverman,

2006). The cellular responses by blood cells (hemocytes) include the recognition, phagocytosis and encapsulation of microbes (Williams, 2007). It has been shown that chronic ethanol exposure can reduce the immune response in mice (Jerrells et al., 1990).

The goal of this study is to test the hypothesis that *Drosophila* larvae which produce mildly elevated levels of endogenous mitochondrial H_2O_2 develop faster under stressful environmental conditions. We identified that larvae harboring the Dahomey mitotype were better able to respond to exogenous stress than those with the Alstonville mitotype due to priming of their antioxidant responses. When fed diet treated with stressors larvae harboring the Dahomey mitotype develop faster into larger pupae and had an increase in antioxidant capacity, with no reduction in membrane potential or RCR. In contrast, Alstonville larvae fed ethanol treated diet had reduced survival after bacterial infection suggesting a complex interaction with the mitotype influences the fly immune response. This study indicates that the primed antioxidant response in Dahomey has potential to provide a benefit in hot climates where fruits rot quickly.

2. Methods

2.1. Experimental conditions

2.1.1. Strains and maintenance

The six fly strains used in this study were studied in pairs and constructed from four mitotypes and two nuclear DNA backgrounds. We refer to these pairs in the form mitotype; nuclear type. The first pair is Dahomey (Dah); w^{1118} and Alstonville (Alst); w^{1118} (Clancy, 2008). The second pair is Madang (Mad); w^{1118} and Victoria Falls (VF); w^{1118} and the third is Dah; CS with Alst; CS (Aw et al., 2018). Dahomey and Alstonville mtDNA have three nonsynonymous differences. In addition to the V161L change in the ND4 subunit of Complex I in Dahomey, there is also a D40N change in the COIII subunit of Complex IV,

and an M185I change in the ATP6 subunit of Complex V. The mitotypes also differ by three rRNA differences (two srRNA changes and one lrRNA change) and 52 A+T-rich region differences (Aw et al., 2018). Madang and Victoria Falls mtDNA have three nonsynonymous changes: V161L in ND4, F148Y in ND2, and M170L in COIII. The Madang and Victoria Falls mitotypes share the same lrRNA bases as Dahomey and Alstonville, respectively. Further, they differ by 41 A+T-rich region differences (Aw et al., 2018). The nuclear backgrounds of w^{1118} and Canton S standardized the nuclear background and tested for possible mitotype by nuclear interactions. w^{1118} was derived from the wild caught Oregon R strain in 1984 (Lindsley, 1968). The Canton S strain was caught in Canton, Ohio around 1916 (Qiu et al., 2017). All fly strains were introduced to a laboratory environment by 2002 (Camus et al., 2012) and are outside the three year timespan in which resistance to stress is rapidly lost under laboratory adaptation (Hoffmann et al., 2001).

To reduce the possibility of accumulated nuclear mutations, females from each of the six strains were crossed to males of their corresponding nuclear DNA background for a minimum of five generations before all assays. To ensure the correct flies were used, flies were genotyped at the beginning and end of all assays. Flies harboring the Dahomey and Alstonville mitotypes were assayed using allele specific PCR (Aw et al., 2018), while those with the Madang and Victoria Falls mitotypes were assayed by Sanger sequencing using ND4 forward 5`-TCTTCGACTTCCAAGACGTTCA-3` and reverse 3`-TGAAGCTCCAGTTTCTGGGTC-5`.

Stock flies were maintained in 250 ml glass bottles at a constant density of 200 ± 25 adults. They were raised on instant *Drosophila* media (Formula 4-24® Instant *Drosophila* medium, Plain, Carolina Biological Supply Company) at 23 °C, with 50% relative humidity, and were kept on 12:12 h light: dark cycles. To produce flies for this study, eggs were collected from 5 d old stock flies using oviposition plates (5% agar, 10% treacle) with a thin

spread of baker's yeast paste. Eggs were collected, washed briefly with diluted bleach, rinsed, and placed onto the experimental diets as per Clancy and Kennington (2001). The microbiome was standardized after 2 d following Aw et al. (2018).

In this study, pupae and late third-instar wandering female larvae were included. Pupae were sexed by the presence of sex-combs (Flagg, 1988). Third-instar larvae migrating on the side of the vial were selected and sexed (Maimon and Gilboa, 2011).

2.1.2. Experimental diets

The 1:2 P:C ratio larval diet was the control (Towarnicki and Ballard, 2017). We used ethanol and H_2O_2 as exogenous dietary stressors. Ethanol is a natural stressor produced by rotting fruit. H_2O_2 is produced in cells as a consequence of OXPHOS but can also be generated through external means including exposure to pollutants and radiation. Following preliminary titrations to optimize concentrations (see Supplementary Fig. S1), a final concentration of 2% was chosen for the ethanol treatment and 2.5 mM for the H_2O_2 treatment. Experimental diets with either ethanol or H_2O_2 were carefully constructed. The 1:2 P:C ratio diet was cooked, cooled to 60 °C in a water bath, and then either ethanol or H_2O_2 was added.

To determine whether ethanol penetrated the gut, larvae from the control and ethanol treatments were collected and larval guts were removed and discarded. The carcasses were placed in 200 µl Eppendorf tubes with a hole pierced in the base using sharpened forceps. Each tube was placed in a 1.5 ml Eppendorf tube, and spun using a desk centrifuge for 10 s to collect hemolymph. From the control and ethanol fed samples, 10 µl of hemolymph was diluted with 50 µl of ddH_2O, added to a 96 well microtiter plate and absorbance was measured at 230 nm. Preliminary analysis showed no difference in absorbance if ddH_2O or

saline was used for the dilution. Hemolymph extracted from six groups of 10 Dah; w^{1118} and Alst; w^{1118} larvae from the control and ethanol diets was assayed.

To determine whether the H_2O_2 added to the diet penetrated the gut, hemolymph was extracted from larvae as above. H_2O_2 levels were measured from the control and treated larvae using Amplex Red. In the presence of peroxidase Amplex Red reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin, which was fluorometrically assayed at 585 nm. Hemolymph extracted from six groups of 10 Dah; w^{1118} and Alst; w^{1118} larvae from the control and H_2O_2 diets was assayed.

2.2. Physiological assays

2.2.1. Time to pupation

Previously, Towarnicki and Ballard (2017) found that Dahomey larvae developed to pupation slower than Alstonville larvae when raised at 23 ºC and fed the 1:2 P:C diet. Eggs were collected as described above and individually placed into vials in batches of 10. Larvae were observed every 4 h during daylight hours and were individually time stamped when they reached pupation. For Dah; w^{1118} and Alst; w^{1118}, 30 replicate vials were established for the control diet, 10 vials for the ethanol treated diet, and 20 vials for the H_2O_2 treated diet. For Mad; w^{1118}, VF; w^{1118}, Dah; CS, and Alst; CS, 10 replicate vials were established for each treatment.

2.2.2. Pupal dry weight

Pupae were collected 4-5 d after pupariation, when sex combs were evident. Female Dah; w^{1118} and Alst; w^{1118} pupae were collected and weighed using a Sartorius microbalance (AGG Gottingen, Germany). Pupae were then placed in 1.5 ml tubes with cotton covering the opening, and dried in an incubator at 60 °C overnight as per Komata et al. (2018). Pupae

were again weighed to record dry weight. For the control diet, 18 groups of 10 pupae were weighed for each mitotype. For the ethanol and H_2O_2 treated diets, 12 groups of 10 pupae were weighed per mitotype.

2.3. H_2O_2 levels and antioxidant responses

2.3.1. H_2O_2 levels

Basal H_2O_2 levels were quantified from isolated mitochondria while total H_2O_2 levels were assayed in the cytosol using Amplex Red (Melvin and Ballard, 2006). Mitochondria and the cytosolic fraction were isolated following Aw et al. (2018). Briefly, larval guts were removed, the carcasses added to mitochondrial isolation buffer, and ground using a pestle. Homogenate was added to a cotton filtered syringe and filtered solution was added to new 1.5 ml Eppendorf tubes. Tubes were then centrifuged to separate the mitochondrial pellet from the supernatant containing the cytosolic fraction. Protein concentrations of the mitochondrial and cytosolic fractions were quantified by Bradford assay. For the Dah; w^{1118} and Alst; w^{1118} mitotypes, late third-instar female larvae were collected in 12 groups of 10 larvae for the control diet, six groups of 10 larvae for the ethanol treatment, and 12 groups of 10 larvae for the H_2O_2 treatment.

2.3.2. Superoxide dismutase (SOD) activity

Mitochondria from Dah; w^{1118} and Alst; w^{1118} were isolated, as reported above, and SOD activity was determined in mitochondria extracts and, in the cytosol, using the ABCAM SOD assay kit (AB65354). Six groups of 10 late third-instar wandering female larvae were assayed for each treatment.

2.3.3. *Expression of antioxidant genes*

Expression of *Sod2* and *GstE1* from Dah; w^{1118} and Alst; w^{1118} was determined from third-instar wandering female larvae. Collected larvae were snap-frozen in liquid nitrogen, RNA was extracted using TRIZOL (Invitrogen), and cDNA was synthesized using SuperScript II RT (ThermoFisher). Primer sequences specific for *Sod2* were obtained from Hu et al. (2013) while those for *GstE1* were sourced from Aw et al. (2018). SYBR Green (ThermoFisher) chemistry was used to perform quantitative real-time PCR (Correa et al., 2012). Following Aw et al. (2018) gene expression was normalized with *Actin* and *RP49* and was expressed as relative to Dah; w^{1118} fed the control diet. For the Dah; w^{1118} and Alst; w^{1118} mitotypes, gene expression was quantified from 12 groups of three larvae for the control diet, and six groups of three larvae for the ethanol and H_2O_2 diets.

2.4. *Mitochondrial functions*

2.4.1. *Membrane potential*

Mitochondria were isolated from Dah; w^{1118} and Alst; w^{1118}, as described above, and mitochondrial membrane potential of isolated mitochondria was fluorometrically quantified by JC-10 dye (Bajracharya and Ballard, 2016). For the control diet, 12 groups of five third-instar wandering female larvae were assayed. For the ethanol and H_2O_2 treatments, six groups of five larvae were tested.

2.4.2. *Respiratory control ratio (RCR)*

RCR from Dah; w^{1118} and Alst; w^{1118} mitochondria was measured using a Seahorse XF24 respirometer (Aw et al., 2018). RCR was calculated as state III / state IVo. For the control

diet, 12 groups of 10 third-instar wandering female larvae were included. For the ethanol and H_2O_2 treated diets, six groups of 10 larvae were assayed.

2.4.3. Microbial challenge

Third-instar wandering female larvae were infected with a sharp needle dipped into a concentrated bacterial solution of *Escherichia coli* (Shia et al., 2009). Between samples the needle was flamed, dipped in room temperature distilled water and then into the bacterial solution. Puncturing of the larval epidermis was confirmed by direct observation of a small discharge of hemolymph. After infection larvae were placed on sucrose plates (5% sucrose, 5% agar) and survival determined after 6 h. When fed the control diet, 50 larvae were assayed for Dah; w^{1118} and 90 for Alst; w^{1118}. When fed food treated with ethanol and H_2O_2, 10 and 40 larvae were assayed for each mitotype, respectively. To determine the effect of injury 10 additional larvae from each mitotype-by-treatment group were poked with a sterile needle. From this control group just two of 60 larvae died so these additional controls are not included in subsequent analyses.

2.5. Data analysis

All data were analyzed for normality using Shapiro-Wilkes W tests and tested for outliers through box plots. If any data points were greater than 1.5 times the interquartile range, they were removed. Mixed-model ANOVA analyses were conducted on all data sets including the main effects of treatment, mitotype and their two-way interaction using JMP 13 (SAS institute). We then conducted *post hoc* Student's t-tests to determine significance between mitotypes. All measurements were from biologically distinct samples forming biological replicates. Statistical tests were not conducted to predetermine sample size.

3. Results

3.1. Physiological assays

3.1.1. Time to pupation

Addition of ethanol and H_2O_2 to the diet caused a flip in development time and Dah; w^{1118} developed more quickly than Alst; w^{1118} (Fig. 1A). In Dah; w^{1118} addition of ethanol to the diet did not influence development time, while addition of H_2O_2 to the diet resulted in development being ~15% faster. Regarding Alst; w^{1118}, dietary ethanol caused development to slow by ~12%, while H_2O_2 caused development to speed up by ~9%. When the mitotypes were harbored in the w^{1118} nuclear background there was a significant effect of mitotype, treatment, and the two-way interaction ($F_{1,499}$= 14.98, $p < 0.001$, $F_{1,499}$= 166.97, $p < 0.001$, $F_{2,499}$= 23.09, $p < 0.001$, respectively). In each condition, *post hoc* t-tests showed significant differences in time to pupation between the mitotypes (control: t_{246}= 3.51, $p < 0.001$, ethanol: t_{87}= 3.57, $p < 0.001$, H_2O_2: t_{166}= 4.68, $p < 0.001$).

Determination of ethanol and H_2O_2 levels in the hemolymph showed the expected results. Absorbance of ethanol was significantly higher in the ethanol fed larvae compared to the controls (see Supplementary Fig. 2A), and significantly higher basal ROS levels were found in the H_2O_2 treated larvae (see Supplementary Fig. 2B).

Permuting the mitotype and nuclear background generalized the observed differences in development time. The Madang mitotype (with the V161L mutation) reacted like Dahomey, while the Victoria Falls mitotype responded in a manner similar to Alstonville (Fig. 1B). ANOVA showed significant effects of mitotype, treatment and their interaction ($F_{1,255}$= 6.84, $p < 0.001$, $F_{1,255}$= 44.49, $p < 0.001$, $F_{2,255}$= 15.56, $p < 0.001$, respectively). Again, *post hoc* t-tests showed significant differences in time to pupation between the mitotypes (control: t_{87}= 2.93, $p = 0.004$, ethanol: t_{86} = 2.91, $p < 0.001$, H_2O_2: t_{82} = 4.68, $p < 0.001$). Permuting the nuclear genetic background with Canton S further corroborated the

influence of the mtDNA mutation (Fig. 1C), with significant effects of mitotype, treatment and their interaction observed ($F_{1,240}$= 6.84, p < 0.001, $F_{2,255}$= 44.49, p < 0.001, $F_{2,255}$= 15.56, p < 0.001, respectively). *Post hoc* t-tests showed significant differences in time to pupation between the mitotypes (control: t_{75}= 2.77, p = 0.007, ethanol: t_{81}= 4.45, p < 0.001, H_2O_2: t_{84}= 3.64, p < 0.001). As a consequence of the generalization we focus on Dah; w^{1118} and Alst; w^{1118} mitotypes for the remainder of the study.

3.1.2. Pupal dry weight

The mitotypes responded differently to the experimental diets. When ethanol and H_2O_2 are added to the food the weight of Dah; w^{1118} pupae increased by ~65% while that of Alst; w^{1118} pupae decreased by ~27% (Fig. 1D). Pupal weight showed a significant main effect of mitotype, no significant main effect of treatment, but a significant mitotype and treatment interaction ($F_{1,78}$= 14.45, p < 0.001, $F_{2,78}$= 1.70, p = 0.19, $F_{2,78}$= 17.28, p < 0.001). *Post hoc* t-tests showed significant differences in pupal dry weight between the mitotypes (control: t_{34}= 3.03, p < 0.001, ethanol: t_{22}= 4.20, p < 0.001, t_{22}= 3.49, p = 0.002). For clarity, we refer to Dah; w^{1118} as Dahomey and Alst; w^{1118} as Alstonville for the remainder of the study.

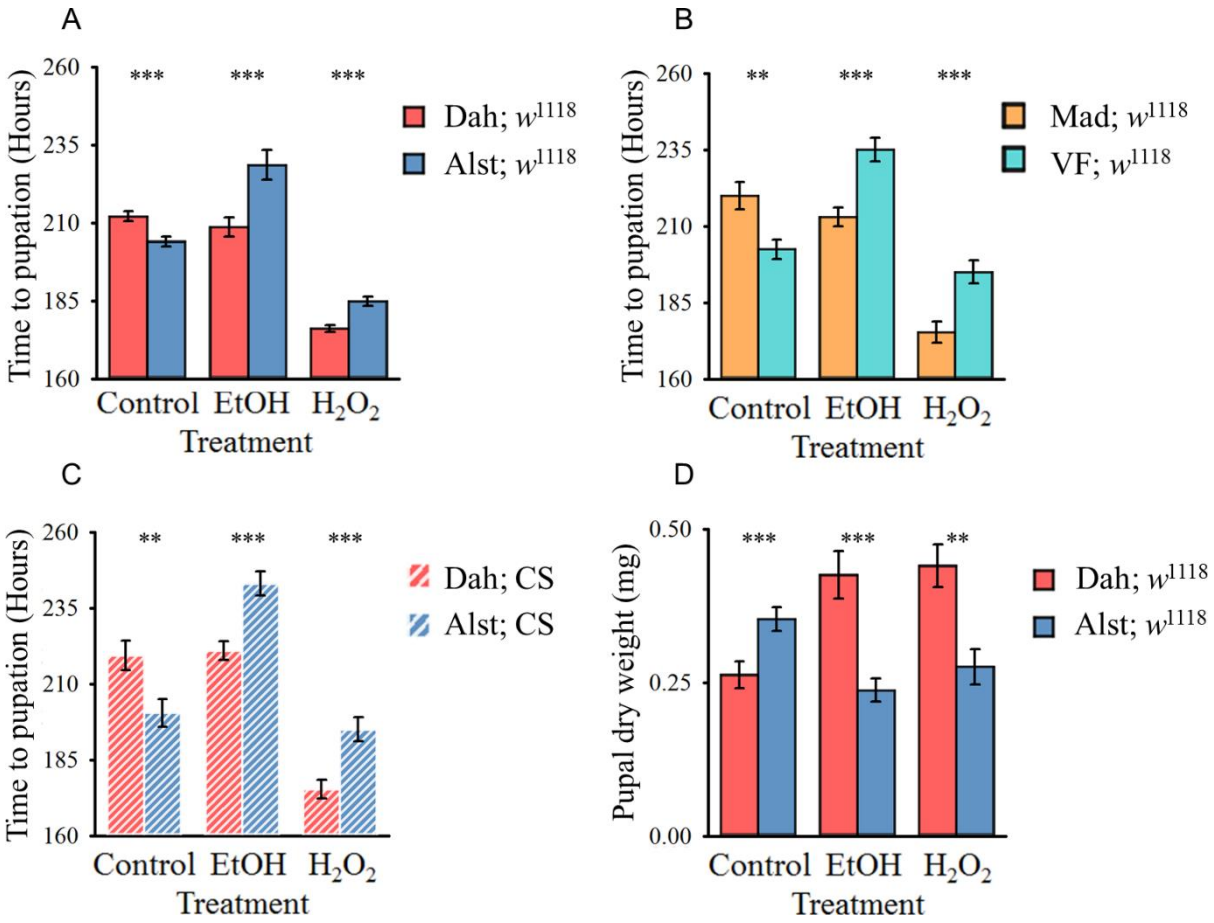


Fig. 1. Physiological assays show that the V161L ND4 mutation in complex I influences time to pupation and pupal weight. (A) Time to pupation of Dahomey (Dah; w^{1118}) and Alstonville (Alst; w^{1118}). (B) Time to pupation of Mad; w^{1118} and VF; w^{1118}. (C) Time to pupation of Dah; CS and Alst; CS. (D) Pupal dry weight of Dahomey (Dah; w^{1118}) and Alstonville (Alst; w^{1118}). Bars show the mean (± SE). Above the bars ** indicates $p < 0.01$, *** indicates $p < 0.001$, as determined by *post-hoc* t tests (see text for details).

3.2 Hydrogen peroxide levels and antioxidant responses

3.2.1. H_2O_2 *levels*

H_2O_2 levels are higher in Dahomey than Alstonville and levels are most similar when larvae are fed the control diet (Fig. 2A). ANOVA of basal mitochondrial H_2O_2 production showed a significant effect of mitotype, but no significant effect of treatment or a mitotype by

354 treatment interaction ($F_{1,30}$= 50.12, p < 0.001, $F_{2,30}$= 0.43, p= 0.65, $F_{2,30}$= 3.17, p = 0.06,
355 respectively). *Post hoc* t-tests showed significant differences in mitochondrial H_2O_2 levels
356 between mitotypes (control: t_{17}= 2.85, p= 0.01, ethanol: t_6= 4.52, p = 0.004, H_2O_2: t_7= 6.28, p
357 < 0.001).

358 Cytosolic H_2O_2 levels were affected by treatment and mitotype. When fed the stress
359 treated diets levels were lower in Dahomey than Alstonville but the reverse was true when
360 larvae were fed the control diet. Dietary addition of ethanol and H_2O_2 increased cytosolic
361 H_2O_2 by ~17% in Dahomey and by ~91% in Alstonville (Fig. 2B). ANOVA showed
362 significant effects of mitotype, treatment, and the mitotype by treatment interaction ($F_{1,46}$=
363 8.43, p < 0.001, $F_{2,46}$= 49.08, p < 0.001, $F_{2,46}$= 18.93, p < 0.001, respectively). *Post hoc* t-tests
364 showed significant differences in cytosolic H_2O_2 between mitotypes in each condition
365 (control: t_{19}= 5.93, P < 0.001, ethanol: t_8= 2.93, p = 0.02, t_{19}= 3.58, p = 0.002).

366

367 *3.2.2. Superoxide dismutase (SOD) activity*

368 Overall, mitochondrial SOD activity was ~71% higher in Dahomey than Alstonville larvae
369 (Fig. 2C). Again, activity was most similar when larvae were fed the control diet. ANOVA of
370 mitochondrial SOD activity showed a significant effect of mitotype, but no significant effect
371 of treatment or mitotype by treatment interaction ($F_{1,24}$= 78.29, p < 0.001, $F_{2,24}$ = 2.49, p =
372 0.10, $F_{2,24}$= 1.96, p= 0.16, respectively). *Post hoc* t-tests showed significant differences
373 between mitotypes (control: t_8= 6.36. p < 0.001, ethanol: t_8= 4.76, p = 0.001, H_2O_2: t_8= 5.27,
374 p < 0.001).

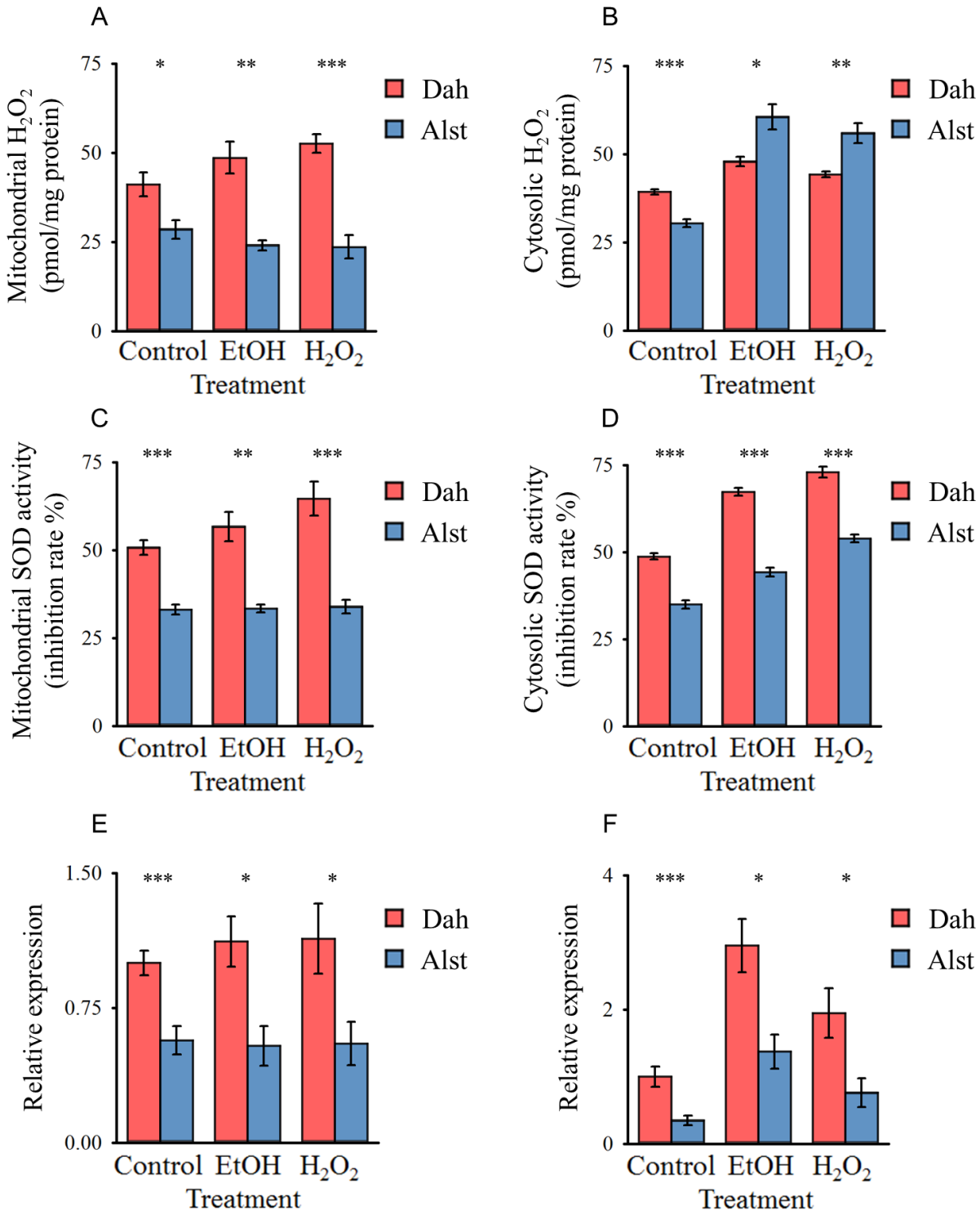
375 Cytosolic SOD activity was ~42% higher in Dahomey than Alstonville larvae.
376 Activity was highest in larvae fed food supplemented with H_2O_2 and lowest in the control
377 group. ANOVA of cytosolic SOD activity showed significant effects of mitotype, treatment,
378 and the mitotype by treatment interaction ($F_{1,24}$= 292.5, p < 0.001, $F_{2,24}$= 134.33, p < 0.001,

$F_{2,24}$= 6.09, p = 0.007, respectively). *Post hoc* t-tests showed significant differences in cytosolic SOD between mitotypes in each treatment (control: t_8= 8.58, p < 0.001, ethanol: t_8= 12.51, p < 0.001, H_2O_2: t_8= 8.78, p < 0.001).

3.2.3. Expression of antioxidant genes

Expression of *Sod2* in Dahomey was about twice that of Alstonville larvae (Fig. 2E) in all treatments. ANOVA of *Sod2* expression showed a significant effect of mitotype, however, treatment and the mitotype by treatment interaction were not significant ($F_{1,40}$= 23.87, p < 0.001, $F_{2,40}$= 0.24, p = 0.79, $F_{2,40}$= 0.65, p = 0.53, respectively). *Post hoc* t-tests showed significant mitotype specific differences in *Sod2* expression in all experimental groups (control: t_{22}= 3.93, p < 0.001, ethanol: t_9= 2.99, p= 0.02, H_2O_2: t_9= 2.39, p = 0.04).

Expression of *GstE1* in Dahomey was more than double that of Alstonville on all diets. Addition of ethanol and H_2O_2 to diet increased *GstE1* expression in both mitotypes 5-fold and 3-fold, respectively (Fig. 2F). ANOVA of *GstE1* expression showed significant effects of mitotype and treatment, but the interaction of mitotype and diet was not significant ($F_{1,38}$= 33.15, p < 0.001, $F_{2,38}$= 22.34, p < 0.001, $F_{2,38}$= 2.25, p = 0.12, respectively). *Post hoc* t-tests showed significant differences in *GstE1* expression levels between the mitotypes (control: t_{21}= 3.88, p < 0.001, ethanol: t_9= 3.12, p = 0.01, H_2O_2: t_8= 2.49, p = 0.04).



397

398 **Fig. 2.** H_2O_2 levels and antioxidant responses of Dahomey (Dah) and Alstonville (Alst)

399 larvae in both the mitochondria and cytosol. (A) Mitochondrial H_2O_2 levels. (B) Cytosolic

400 H_2O_2 levels. (C) Mitochondrial SOD activity. (D) Cytosolic SOD activity. (E) Expression of

401 *Sod2*. (F) Expression of *GstE1*. Bars show the mean (± SE). Above the bars * indicates p <

0.05, ** indicates $p < 0.01$, *** indicates $p < 0.001$ as determined by *post-hoc* t-tests (see text for details).

3.3. Mitochondrial functions

3.3.1. Membrane potential

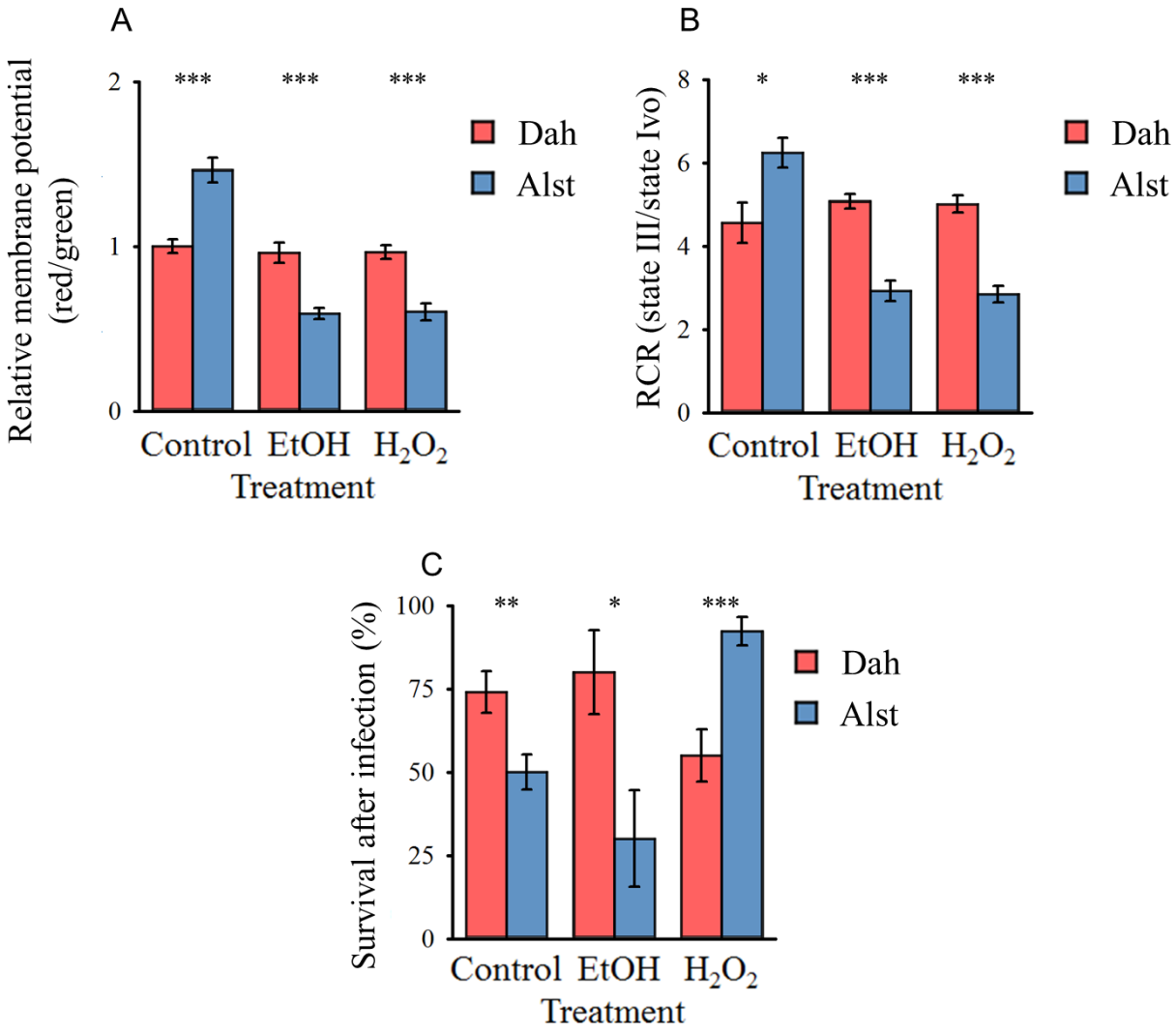
Membrane potential appeared to be buffered to dietary treatment effects in Dahomey but not Alstonville larvae. Dietary addition of ethanol and H_2O_2 did not affect the membrane potential of Dahomey larvae but caused a ~59% reduction in the mitochondrial membrane potential of Alstonville larvae (Fig. 3A). ANOVA of mitochondrial membrane potential showed no significant effect of mitotype but significant effects of treatment, and the interaction of mitotype and treatment ($F_{1, 37}= 1.94$, $p = 0.17$, $F_{2,37}= 36.08$, $p < 0.001$, $F_{2,37}= 27.33$, $p < 0.001$, respectively). Further, *post hoc* t-tests showed significant differences in membrane potential between Dahomey and Alstonville in each condition (control: $t_{19}= 5.02$, $p < 0.001$, ethanol: $t_{10}= 4.81$, $p < 0.001$, H_2O_2: $t_8= 4.92$, $p < 0.001$).

3.3.2. Respiratory control ratio (RCR)

Like membrane potential, RCR is not affected by stressors in Dahomey. In contrast, dietary addition of ethanol and H_2O_2, caused the RCR of Alstonville to decrease by 54% (Fig. 3B). ANOVA of RCR showed significant effects of mitotype, treatment and the interaction of mitotype and treatment ($F_{1,42}= 5.97$, $p = 0.02$, $F_{2,42}= 8.97$, $p < 0.001$, $F_{2,42}= 16.06$, $p < 0.001$, respectively). *Post hoc* t-tests showed significant differences in RCR between the mitotypes in each treatment (control: $t_{22}= 2.7$, $p = 0.01$, ethanol: $t_{10}= 4.48$, $p < 0.001$, H_2O_2: $t_{10}= 6.91$, $p < 0.001$).

3.3.3. Microbial challenge

The influence of microbial challenge on larval survival was treatment and mitotype dependent (Fig. 3C). In the case of Dahomey, addition of ethanol to the diet resulted in 80% of larvae surviving. Addition of H_2O_2 reduced survival to 55%, while 74% survived on the control diet. Comparatively, Alstonville larvae are more treatment sensitive. Addition of ethanol to the diet resulted in 30% larval survival, with the high error on this sample due to the small sample size. Addition of H_2O_2 to the diet increased survival to 92% compared to the 50% survival observed on the control diet. ANOVA of survival after microbial challenge showed no significant main effects of mitotype or treatment, but a significant mitotype by treatment interaction ($F_{1,233}= 2.3$, $p = 0.13$, $F_{2,233}= 2.17$, $p = 0.12$, $F_{2,233}= 13.71$, $p < 0.001$, respectively). *Post hoc* t-tests showed significant differences in survival after microbial challenge between the mitotypes in each treatment (control: $t_{138}= 2.82$, $p = 0.006$, ethanol: $t_{18}= 2.47$, $p = 0.02$, H_2O_2: $t_{77}= 4.09$, $p < 0.001$).



439

440 **Fig. 3.** Mitochondrial functions and microbial challenge of Dahomey (Dah) and Alstonville
441 (Alst). (A) Mitochondrial membrane potential relative to Dahomey on the control diet. (B)
442 RCR as determined by state III respiration over state IVo respiration. (C) Percentage survival
443 after infection with *E. coli*. Bars show the mean (± SE). Above the bars * indicates $p < 0.05$,
444 ** indicates $p < 0.01$, *** indicates $p < 0.001$ as determined by *post-hoc* t-tests (see text for
445 details).

446

447

4. Discussion

Insects live in heterogeneous environments and they must be able to survive a battery of environmental stressors if they are to reproduce. ROS are chemicals that have been shown to be involved in a range of responses to stressful environments. Our knowledge of the effects of ROS is still growing but it is clear that levels must be balanced within an organism for normal survival and reproduction. At low levels ROS is a signaling molecule and can promote cellular proliferation, thiol peroxidase functions, and influence gene expression (D'Autréaux and Toledano, 2007; Ristow and Zarse, 2010; Stone and Yang, 2006). However, excess ROS levels cause damage to cellular components and organelles (Circu and Aw, 2010; Redza-Dutordoir and Averill-Bates, 2016; Simon et al., 2000). This necessary balance prompted us to consider mechanisms by which ROS is managed at a cellular level. We hypothesized that high levels of mitochondrial H_2O_2 would result in ROS leaking into the cytosol through RIRR. Plausibly, the elevated cytosolic ROS levels would then induce a cytosolic antioxidant response and prevent damage from dietary toxicants that enter cells. To test this hypothesis, we compared the organismal and cellular responses of larvae harboring two *Drosophila* mitotypes when they were fed diets supplemented with two stressors. These mitotypes differ by a V161L ND4 mutation that causes differences in mitochondrial ROS production (Aw et al., 2018).

We identified that the Dahomey mitotype developed to pupation faster than the Alstonville mitotype when stressors were added to diet (Fig 1A). Pupae were also heavier (Fig 1D). However, there are specific differences and similarities in the action of the two stressors on mitotype development. Addition of ethanol to the diet did not change the time to pupation of Dahomey but slowed the development of Alstonville. In contrast, dietary addition of H_2O_2 sped up the development of both mitotypes. As previously reported, when fed the control diet, the Dahomey mitotype developed slower than the Alstonville mitotype (Aw et

al., 2018) and had reduced pupal weight (Fig. 1D). Developing to sexual maturity in insects is a key fitness trait (Boivin et al., 2001; Feng et al., 2009; Kliot and Ghanim, 2012), where faster development is correlated with higher adult fecundity and adult survival (Kingsolver and Huey, 2008). Pupal weight is positively linked with mating success and fecundity of the adult fly (Angilletta Jr et al., 2004; De Moed et al., 1999; Kingsolver and Huey, 2008) and suggests a possible adaptation of Dahomey mtDNA to stressful environments. Differences and similarities in response to ethanol and H_2O_2 stress have previously been observed. Logan-Garbisch et al. (2014) found differences in the response to the two stressors. They identified that a *D. melanogaster* strain with mutations in *Phosphoinositide-dependent kinase 1* displayed increased survival to adulthood when fed ethanol treated diet but had decreased survival when fed H_2O_2 treated diet. Courgeon et al. (1993) found that acute exposure of *D. melanogaster* cells to ethanol and H_2O_2 stress increased the rate of actin synthesis at similar levels for both stressors. Heterogeneity in larval preferences of *D. melanogaster* strains to agar containing alcohol has also been reported (Parsons, 1977) and a future study may test whether the Dahomey and Alstonville larvae have distinct preferences to agar containing alcohol.

We identified a mitotype dependent beneficial effect of elevated basal mitochondrial H_2O_2 in promoting an antioxidant response to the two exogenous stressors. In all treatments, Dahomey larvae had higher mitochondrial H_2O_2 levels than Alstonville and these levels were correlated with the mitochondrial antioxidant response, as measured by SOD activity and *Sod2* expression (Fig 2A, C & E). While low levels of mitochondrial ROS are often considered ideal (Stone and Yang, 2006), this may only be true under laboratory conditions. Perhaps, this view comes from the literature suggesting that mitochondrial ROS is a by-product of OXPHOS and is often considered indicative of reduced coupling (Bazil et al., 2016; Fruehauf and Meyskens, 2007; Marcinek et al., 2005). Plausibly, however, mildly

498 elevated levels of mitochondrial ROS may provide flexibility to environmental stressors by
499 priming the antioxidant response. Ristow and Schmeisser (2014) described increased
500 mitochondrial ROS levels as causing a vaccination-like adaptive response that provides long
501 term stress defense. Mitochondrial ROS have also been implicated in providing increased
502 survival under hypoxia in *Caenorhabditis elegans* (Schieber and Chandel, 2014) and in
503 maintaining organismal homeostasis in a variety of organisms (Shadel and Horvath, 2015).
504 When fed the stressful diets, Dahomey had lower cytosolic H_2O_2 than Alstonville
505 (Fig. 2B), while having mildly higher levels when fed the control diet. The mildly higher
506 levels in larvae fed the control diet are likely due to RIRR leakage into the cytoplasm which
507 then induced a cytosolic antioxidant response. Fed the stressful diets Dahomey had higher
508 cytosolic SOD activity and higher expression of *GstE1* than Alstonville (Fig. 2D & F). In
509 contrast, we suggest the high levels of cytosolic H_2O_2 in Alstonville fed the stressors occurred
510 because the antioxidant system was not primed by RIRR from the mitochondria. If true, this
511 suggests that RIRR is an important mechanism that has potential to influence fitness of
512 organism harboring distinct mitotypes. In addition to the tested antioxidants, it is possible that
513 a wider range of antioxidants are upregulated in Dahomey as part of a response to elevated
514 mitochondrial H_2O_2 levels (Ristow and Zarse, 2010; Yun and Finkel, 2014). In *Drosophila*,
515 Keap1/Nrf2 signaling is activated by oxidants, inducing antioxidant and detoxification
516 responses, and confers increased tolerance to oxidative stress (Sykiotis and Bohmann, 2008).
517 Similar antioxidant responses are associated with improved health and fitness in a variety of
518 organisms (Shirpoor et al., 2009; Wentzel and Eriksson, 2006; Wentzel et al., 2006; Zhang et
519 al., 2016). Zhang et al. (2016) found that the freshwater snail *Radix swinhoei* sensitively
520 responds to toxins by manipulating its antioxidant system to cope with toxicity. Shirpoor et
521 al. (2009) showed that ethanol intake by pregnant Wistar rats induces homocysteine-mediated
522 oxidative stress in the offspring that can be alleviated by vitamin E as an antioxidant. Wentzel

et al. (2006) found that ethanol exposure in mice disturbs embryogenesis partly by enhanced oxidative stress, and the adverse effects can be ameliorated by antioxidative treatment. We suggest future studies may investigate the antioxidant response further through measurement of catalase and thiol levels.

The primed cytosolic antioxidant response of Dahomey likely prevented damage to mitochondrial membranes from the dietary ethanol and H_2O_2. Fed the stressful diets mitochondrial membrane potential and RCR were unaltered in Dahomey but decreased in Alstonville. Membrane potential is the key bioenergetic factor that controls the respiratory rate and ATP synthesis (Nicholls, 2004) and is reduced by proton leak (Korshunov et al., 1997; Skulachev, 1996). RCR is considered the most useful general measure of mitochondrial function, as changes that reduce OXPHOS are reflected in reductions of RCR (Affourtit and Brand, 2005; Brand and Nicholls, 2011).

There were distinct differences in responses of the two mitotypes to bacterial infection. Overall, more Dahomey survived infection and their response to stressors was less variable than Alstonville. Furthermore, the relative responses of the mitotypes to H_2O_2 differed when compared to the ethanol treatment and the control. Notably, a high proportion of larvae survived infection. It is well established that H_2O_2 has antibacterial properties, and can be localized to the site of bacterial infection (Brun et al., 2006; Fang, 2011; Spooner and Yilmaz, 2011). Likely, the immune deficiency pathway is moderated by ROS levels. West et al. (2011a) revealed a novel pathway linking innate immune signaling to mitochondria and implicates ROS as important components of antibacterial responses. This idea of a protective role of H_2O_2 does not, however, explain the ethanol treated diet results where Alstonville has high H_2O_2 levels but low survival after infection. Plausibly, then there may be a specific mitotype by ethanol interaction response to bacterial infection. In *Drosophila*, Zhu et al. (2014) show that complex interactions between the mitotype, nuclear genome, and the

environment influence cellular and organismal functions that affect fitness, aging, and disease in nature. Additional research is required to determine whether these survival results are specific to the interaction of ethanol and *E.coli* or if they are generalizable to additional pathogens (Brun et al., 2006; Lemaitre et al., 1996).

In conclusion, we identified a mitotype specific response to environmental stress, whereby the mitotype that produces slightly elevated levels of endogenous mitochondrial H_2O_2 is resistant to exogenous dietary stressors. We show that the Dahomey mitotype developed to pupation faster than the Alstonville mitotype when ethanol and H_2O_2 were added to diet. This coincided with no reduction of mitochondrial functions in Dahomey, while the presence of dietary stressors reduced membrane potential and RCR in Alstonville. We argue the high levels of endogenous H_2O_2 production in Dahomey, due to the mutation in the ND4 subunit of Complex I, primed the antioxidant response, as seen by the higher activity of cytosolic SOD and expression of *GstE1*. We propose that a primed antioxidant response may provide a mitotype dependent resilience, or even organismal preference, to exogenous dietary ethanol stress, particularly in climates where food rots more quickly.

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The study was designed by SGT and JWOB. SGT and LK performed assays and analyzed the data. SGT wrote the first draft of the manuscript, and JWOB contributed substantially to revisions, placed the work into a theoretical context and funded the project.

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